

The background of the cover is a high-magnification electron micrograph. It shows a large, rounded cell at the top, likely an oocyte, with a textured surface. Below it is a dense cluster of smaller, rounded cells, possibly endometrial cells or early-stage embryos, also with a granular texture. The overall color is a dark, mottled brown with some lighter, fibrous-looking structures interspersed.

Electron Microscopy of Human Oocytes,
Cleavage Stage Ova and Pre-Implantation
Endometrium within an In Vitro
Fertilization Programme

by
Per Sundström
Malmö 1984

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From the Department of Obstetrics and Gynecology, University of Lund,
Malmö General Hospital, S-214 01 Malmö, Sweden and Department of Human
Anatomy, University of Uppsala, BMC, S-751 23 Uppsala, Sweden

ELECTRON MICROSCOPY OF HUMAN OOCYTES, CLEAVAGE STAGE
OVA AND PRE-IMPLANTATION ENDOMETRIUM WITHIN AN
IN VITRO FERTILIZATION PROGRAMME

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Per Sundström

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...skaffade han sig efter någon väntan ett människofrö, sedan han under mikroskopet konstruerat en couveuse, som kunde hållas vid trettiosex till fyrtioen graders värme. När befruktningen verkställdes, såg han harnarne svärma kring den orörliga honan, vilken han inbillade sig kunna se rodna. Och nu trängdes de, knuffades, piskade varandra i kampen om att få ge impulsen åt ett släkte, fortplanta hans anlag, ympa hans livliga, alstringsrika ande på ett kraftigt, vilt underlag. Men det var icke de grövsta, de med stora, dumma huvuden och tjocka svansar, utan de kvickaste, smidigaste, de eldigaste, som först genomträngde membranen för att få tränga in till kärnan.

August Strindberg
"I havsbandet" 1890

To Birgitta
Gustav, Johanna and

The present thesis is based on the following publications:

- I. Sundström P, Nilsson BO, Liedholm P, Larsson E: Ultrastructural characteristics of human oocyte maturation in ovarian-stimulated cycles (submitted).
- II. Sundström P, Nilsson BO, Liedholm P: Cleavage rate and morphology of early human embryos obtained after artificial fertilization and culture. *Acta Obstet Gynecol Scand* 60:109-120, 1981.
- III. Larsson E, Nilsson BO, Sundström P, Widéhn S: Morphological and microbiological signs of endogenous C-virus in human oocytes. *Int J Cancer* 28:551-557, 1981.
- IV. Sundström P: Interaction between human gametes in vitro. A scanning electron microscopic study (in press).
- V. Sundström P, Nilsson BO, Liedholm P: Scanning electron microscopy of human preimplantation endometrium in normal and clomiphene/human chorionic gonadotropin-stimulated cycles. *Fertil Steril* 40:642-647, 1983.

In the text the papers are referred to by their Roman numerals.

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ABBREVIATIONS

IVF	In Vitro Fertilization
ER	Egg Re-placement
TEM	Transmission Electron Microscopy
SEM	Scanning Electron Microscopy
hCG	human Chorionic Gonadotropin
hMG	human Menopausal Gonadotropin
RT	Reverse Transcriptase
FSH	Follicle Stimulating Hormone
LH	Luteinizing Hormone
GV	Germinal Vesicle
SER	Smooth Endoplasmic Reticulum
MV	Mitochondria-Vesicle (complex)
RNA	Ribonucleic Acid
DNA	Deoxyribonucleic Acid

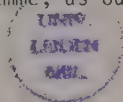
INTRODUCTION

In vitro fertilization (IVF) and egg re-placement (ER) has over the last few years achieved recognition as a clinical complement to tubal surgery and is the only alternative once the tubes have been removed. The quite rapid advances in the field of human IVF have been made possible by extensive work on laboratory animals and in veterinary medicine (100).

The first successful attempt to transfer a cleaving egg from one animal to another, with subsequent development and birth of a fetus, was made in the rabbit by Heape in 1890 (36). In 1944, Rock & Menkin reported that they had achieved fertilization and cleavage of three human oocytes in vitro (70). However, it was not until 1969 that fertilization of a human oocyte in vitro could with certainty be demonstrated, in that a sperm axon was found in the ooplasm close to one of the pronuclei (9). The first human birth following IVF was reported by Steptoe & Edwards in 1978 (84). Since then, many babies conceived by the same method have been born - more than one hundred, to date.

Modern IVF technique has developed from studies in rodents, as they are easy to handle and control (17,68). Compositions of culture media, osmolality and pH, as well as other environmental factors for supporting embryonic growth, have been tested and extensive research conducted in oocyte maturation, cleavage stage ova, and blastocyst implantation. Although knowledge from other species is an essential basis, it cannot always be applied directly in the human situation. Hence, it is necessary to conduct basic research on human gametes, their interaction and on implantation. Clinically, this may contribute to a better understanding of the infertile couple as well as to new contraceptive methods. Generally speaking, research can advance our understanding of human reproductive biology and pathology.

Studies on the ovum, however, are far more difficult to design for the human than for laboratory animals. Legal and ethical aspects limit the availability of human material (22). Nevertheless, oocytes unsuitable for replacement into the woman occasionally become available within an IVF programme and can be taken for research. Furthermore, specimens became available before the start of our IVF programme, as our policy was to form a



scientific basis for the IVF procedures, for instance by studying the morphology and genetics of oocytes and early cleavage stages. These ova were obtained mainly from women operated upon for tubal repair. Donors of the gametes gave their full consent and approval was accorded by the local ethical committee.

Since oocyte maturation, gamete interaction, development of early cleavage stage ova and implantation are some of the areas which are not only poorly understood but also seem to be easily disturbed in an IVF programme, the present work was focused on these events. In approaching these problems, ultrastructural studies by transmission electron microscopy (TEM) and scanning electron microscopy (SEM) were used for the present investigations.

Variations in the degree of oocyte maturation and subsequently in fertilizability of collected oocytes might account for some of the 20 to 70% fertilization failures of IVF (89). It is important to learn the characteristics of the mature oocyte and to be able to mature an oocyte in vitro. This would enhance the prospect of fertilizing several oocytes and establishing a pregnancy (95). Judging oocytes by their ultrastructure is one way to determine what changes are occurring during maturation and whether oocytes which do not become fertilized in IVF attempts show any traits in common.

Type C-virus-like particles in human oocytes were described by us in 1981 (57,62). This feature could have implications for IVF, among other areas. Therefore, work was focused on attempts to obtain further evidence of the presence of C-virus by analysing reverse transcriptase, a virus-coded enzyme, in follicular fluids.

Disorders in sperm attachment onto the zona pellucida can also cause fertilization failure (11). In vivo, a mere few hundred selected spermatozoa reach the ovulated oocyte at the site of fertilization in the Fallopian tube (2), whereas in vitro, several hundred thousand unselected spermatozoa are placed in the vicinity of the oocyte. Many of these spermatozoa are morphologically abnormal (31), which might be hazardous for the fertilization. In the scanning electron microscope, spermatozoa attached

to the oocyte can be examined in great detail and thereby judged morphologically.

Disturbances in the early cleavage stages, which can result in implantation failure following ER, may well be a reality, since ova develop for varying periods of time in an artificial environment (13). On the assumption that the morphology of the early cleavage stages reflects their status after culture, electron microscopy was used to evaluate the procedure.

The implantation rate after ER is on the whole rather low, viz. about 10 to 30% of all ER (90,100). Since only stimulated cycles are used at IVF, hormonal stimulation might influence endometrial development. The ultrastructure of the postovulatory surface epithelium, judged by SEM, can be informative about the structures that first establish contact with the egg after replacement, and of endometrial surface development during peri-implantation.

The AIMS of this thesis were, therefore:

- 1) to study maturing and mature oocytes and cleavage stages by TEM, in order to describe their ultrastructure and to look for characteristic signs of oocyte maturation and of egg development in vitro;
- 2) to study signs of expression of type C-virus-like particles in oocytes by TEM and by virus-related enzymatic activity in follicular fluids;
- 3) to study gamete interaction by SEM in order to determine the number and assess the morphology of spermatozoa attached to the zona pellucida;
- 4) to study by SEM the postovulatory surface endometrial epithelium in unstimulated and stimulated women in order to ascertain whether there are consistent structural differences within and between the two groups of women during the peri-implantation period.

MATERIAL AND METHODS

Clinical Procedures

The material in the studies was obtained from women admitted to the hospital because of infertility, due mainly to damage of the Fallopian tubes, or for legal sterilization. Oocytes and cleavage stage ova which were analysed ultrastructurally were selected at random from women not included in the ER program. Furthermore, oocytes unsuitable for replacement, i.e. uncleaved and degenerating oocytes, were taken for study.

Oocytes are difficult to obtain in unstimulated cycles, especially preovulatory ones. Therefore, in the main, stimulated cycles were used, as they are far easier to regulate and more than one preovulatory oocyte is usually obtained. To induce growth of several follicles, clomiphene citrate, 50 to 150 mg for 5-7 days, was usually given and oocytes were aspirated at midcycle 34-36 hours after an injection of 4,000-6,000 IU of human chorionic gonadotropin (hCG). In unstimulated cycles, the operations were performed at midcycle. Follicle aspiration was carried out at laparoscopy or laparotomy with the woman under general anaesthesia. The follicular fluids were surveyed for oocytes in a stereo-microscope within 5 min of follicle aspiration and a number of oocytes were taken. The fluids were then centrifugated and the supernatant frozen (-20°C) until required for analysis.

Oocytes were classified clinically as preovulatory, intermediate, or immature, according to their appearance in the stereo-microscope (29,45). Preovulatory oocytes are those which are surrounded by a large viscous, dispersed cumulus mass, whereas intermediate oocytes are surrounded by a small and moderately dispersed cumulus mass, and immature oocytes by, at the most, corona cells only, i.e. from denuded to several layers of corona cells.

Some oocytes were fixed immediately after recovery and the rest were placed in bicarbonate-buffered culture medium, supplemented with sera and antibiotics, either in a Petri dish and covered by liquid paraffin, or in a test tube without paraffin, until fixation or incubation with spermatozoa. Incubation and culture of oocytes took place in an incubator (37°C)

gassed with 5% CO₂ in air, or else 5% CO₂, 5% O₂ and 90% N₂. pH was checked by a colour indicator (phenol red) in the medium during routine culturing and by analysing the medium in a pH gauge at irregular intervals. Osmolarity in the culture media used was about 285 mmol/l.

A fresh sperm sample for IVF was obtained on the morning of oocyte collection. Following liquefaction, the sperm sample was washed in culture medium by means of stepwise centrifugation. After dilution, 200,000 or 500,000 spermatozoa were added to each oocyte, either immediately or after 5 hours of incubation (91) of the oocytes. Care was taken not to expose the oocytes to room atmosphere too often and examinations were consequently made infrequently during the periods of incubation and culture. About 24 hours after incubation of the oocytes in sperm suspension, the culture medium was changed and the oocytes examined for pronuclei and polar bodies. Fertilization was presumed to have occurred when two pronuclei were observed in the centre of the ooplasm within 24 hours and the oocytes subsequently cleaved (102).

Postovulatory endometrial biopsies were collected from unstimulated women and from women stimulated with clomiphene (50 mg for 5 days) and hCG (6,000 IU). The biopsy sample was collected, with the woman under general anaesthesia, from the anterior wall of the upper part of the uterine cavity. The specimens were dated according to the change in plasma hormone level of progesterone or of progesterone and oestradiol-17 β . Plasma hormones were analysed by conventional radio-immunoassay. The day of ovulation was assumed to be the day after the peak of oestradiol-17 β coinciding with a rise - from the basal level - in the progesterone level or, in a few cases, the day of the first pronounced increase in the serum progesterone level (61,102). Furthermore, in some of the stimulated women, follicle aspiration had been carried out at mid-cycle in the same menstrual cycle as the biopsy collection. From a few of these women, preovulatory oocytes which cleaved after fertilization in vitro were obtained, thereby establishing the time of ovulation more accurately.

Preparatory Procedures

Fixation of uncleaved oocytes and cleavage stage ova, from the 4-cell

to the 10-12-cell stage, was done by placing the specimens in a solution of 2.5% glutaraldehyde in culture medium without serum (room temperature, pH 7.4).

The appearance of fixed specimens was judged under the light microscope. In uncleaved oocytes, the presence of polar bodies and pronuclei as well as the general appearance of the ooplasm were noted. Atretic oocytes showed a dense retracted ooplasm (78), while normal-appearing ones showed a homogeneous non-retracted ooplasm. In cleaved ova, the cleavage stage and the general appearance of individual blastomeres were noted. The cytoplasm of blastomeres appeared translucent and homogeneous. Blastomeres could differ slightly in size. The number of spermatozoa on the zona was estimated roughly for later comparison with the number observed in the scanning electron microscope.

Preparation of oocytes and cleavage stages for TEM:

After fixation, the specimens were rinsed in phosphate buffer, post-fixed in osmium tetroxide, dehydrated in increasing concentrations of ethanol and embedded in Epon 812. Sections were stained with uranyl acetate and lead citrate. Sections from two to four levels of each specimen were examined in the electron microscope. Oocytes were regarded as atretic when TEM revealed extensive vacuolization and condensation of the ground substance and pre-atretic when numerous vacuoles were seen in the centre of the ooplasm.

Analysis of follicular fluids for reverse transcriptase (RT) activity:

Assays for RT activity were made according to standard methods (72). Briefly, particulate material in the follicular fluid was fractionated on a continuous sucrose gradient and aliquots from each fraction at densities between 1.15 and 1.20 g/cm³ were tested for RT activity by using radioactive nucleotides and primer-template complexes.

The acid-insoluble radioactivity was collected on a Millipore filter, dried and counted in a liquid scintillation counter. An enzymatic activity of more than twice the background was considered positive.

Preparation of ova and corona cells for SEM:

After fixation, the specimens were rinsed in distilled water and placed on polylysine-L prepared coverslips (49). Acetone was used in increasing concentrations for dehydration, prior to critical point drying. The dried specimens were coated with gold-palladium by ion sputtering prior to analysis in a scanning electron microscope.

Preparation of endometrial biopsies for SEM:

The endometrial biopsies (2-3 cm x 2-3 mm) were fixed immediately in a solution of 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). After fixation, each biopsy specimen was cut into small pieces under distilled water and dehydrated in increasing concentrations of acetone, prior to critical point drying. Five or six pieces from each biopsy specimen were mounted on metal stubs and covered with gold-palladium by ion sputtering, prior to examination by SEM.

MATURING OOCYTES AND CLEAVAGE STAGES

Oocyte maturation comprises: 1) chromatin condensation in the germinal vesicle, 2) breakdown of the germinal vesicle nuclear membrane, 3) formation of the first meiotic spindle, 4) first meiotic division and extrusion of the first polar body, 5) formation of the second meiotic spindle, 6) arrest of meiosis at second meiotic metaphase (50,93) (Fig. 1). At this stage, the oocyte is ovulated and fertilizable for an unknown period of time (20,82). However, the fertilizable life span of, for instance, the hamster oocyte is measurable in hours after ovulation (104). If fertilization does not occur, the oocyte degenerates and the corpus luteum regresses into a non-active corpus albicans.

An oocyte which has commenced germinal vesicle breakdown is called a maturing oocyte and defined as mature when it has reached the second meiotic metaphase (93) (Fig. 2). Oocytes acquire the ability to resume meiotic maturation during their final stage of growth and follicular development. However, resumption of meiosis differs significantly *in vivo* versus *in vitro* (50).

In vivo, resumption of meiosis is triggered by the luteinizing hormone (LH) and includes changes in follicular production of steroids, prostaglandin, cyclic AMP, and in the integrity of intercellular junctions between the granulosa cells and the oocyte (50,71,93). The hormonal control of development of meiotic competence of an oocyte in the early antral stage requires the influence of the follicle-stimulating hormone (FSH) and of oestrogen. Granulosa cell projections extending into the oocyte indirectly regulate the antral oocyte and keep it in its resting stage of meiosis, i.e. in the germinal vesicle (GV) stage. The release from inhibition is mediated by LH. However, LH is not the prime trigger factor for resumption of meiosis, but acts rather via receptors on granulosa cells, thereby preventing their inhibitory effect (50). Oocyte maturation inhibitor (OMI) is one of probably several inhibitors involved (92). Cyclic AMP may be another, but it may also act in a promotory capacity. Final maturation of the oocyte may be dependent on follicular fluid steroids, but their role has been debated (93).

Follicular fluid hormone contents of oestrogen and progesterone have been shown to rise to critical levels in preovulatory follicles (29,88, 102). The hormone levels in the follicle probably reflect the maturation stage of the oocyte (102) and high levels are believed to protect the oocyte from premature atresia prior to ovulation (81).

Why only some oocytes are selected for maturation and why usually only one oocyte becomes fully matured and ovulated, while the rest are destined for atresia, is not known. However, follicular growth is a continuum and one follicle may be more ready to respond to growth stimuli than others. Those oocytes which have commenced maturation but are not ovulated, regress when left in situ, though atresia can occur at any stage of oocyte maturation (23,82).

In vitro, oocytes resume meiosis spontaneously in rather simple culture media probably because some inhibitory agent in the follicular fluid and in granulosa cells is removed when the oocyte is transferred into the culture medium (67) or due to changes in the oocyte upon liberation (30). An oocyte in the GV stage has matured after about 36 hours of incubation in vitro (23).

Since in vitro matured human oocytes (from the GV stage and later) have been the origin of IVF children (95), it is evident that viable cleaving eggs can be obtained from both preovulatory and in vitro matured oocytes.

Fertilization requires both a mature oocyte and a capacitated and acrosome reacted spermatozoon (23). Soon after sperm penetration into the oocyte, the cortical granules are released, inducing the zona reaction and blocking polyspermia (75). Initiation of the second meiotic division is triggered by the fertilizing spermatozoon and the second polar body is extruded shortly after fertilization (Figs.1,2). The whole of the spermatozoon is engulfed by the cytoplasm. The sperm head then decondenses and a male pronucleus forms within a few hours. Simultaneously, the female pronucleus is formed. The two pronuclei move towards the centre and their nuclear membranes break down. The chromosomes condense and combine on the first cleavage spindle.

The ovum divides mitotically, thereby doubling the cell number at each cleavage. The cleaving ovum, whose cells become smaller and smaller with each cleavage, is enclosed in its zona pellucida while being transported along the Fallopian tube (Fig. 3). This journey takes about 3 to 4 days (18). The egg sheds its zona pellucida after reaching the uterine cavity. Implantation of the egg, now called a blastocyst, takes place on the 6th to 7th day following ovulation. The pregnancy is established and proceeds under the control of a signal system functioning between the cleaving egg and the host (35,51,69).

The ultrastructure of the various maturation and cleavage stages of in vivo and in vitro developed oocytes has been studied (4,6,38,39,63,73,76, 83,87,107, 108). Maturation in vivo is characterized by a general increase in number of cytoplasmic organelles, with the exception of annulate lamellae, and by the development of microvilli on the cell membrane and formation of cortical granules in the ooplasm. When comparing oocytes developed in vivo vis-à-vis in vitro it was found that the cultured oocytes seemed to lack a well-developed Golgi apparatus, but that they had acquired spheroidal aggregates consisting of flattened tubuli of the smooth endoplasmic reticulum surrounded by mitochondria (108). Furthermore, after incubation of preovulatory oocytes for a few hours in vitro, cortical granules were found to increase markedly in number (74). Otherwise, no cytoplasmic structure showed any consistent and regular change during oocyte maturation, whether matured in vivo or in vitro.

Published ultrastructural descriptions of cleavage stage ova are few. One cleaved human ovum in the 7-cell stage, developed in vivo, was described in 1978 (66). A cleaving egg developed in vitro may differ in appearance from one developed in vivo and this could have implications for the outcome of the IVF/ER procedure.

The ultrastructure of in vitro fertilized and developed ova was described for the first time by us in 1980 (86). The stages examined were those at which an egg is usually replaced into the recipient. Our findings are now presented in the following text. Later studies by other investigators on in vitro developed early cleavage stages confirmed our observations (21,46,77). Furthermore, abnormal features have been described, for in-

stance in the form of multinucleated blastomeres or fragmentation (46,62, 77).

RESULTS OF PRESENT INVESTIGATION AND DISCUSSION - Paper I-II

The perivitelline space was narrow and numerous microvilli from the cell membrane were projecting into this space in oocytes. In blastomeres, however, the cell membrane showed a smoother outline.

A few corona cells have been found trapped in the perivitelline space of an immature oocyte. The cells observed differed in appearance from corona cells located on the outside of the zona pellucida, in that numerous long and slender microvilli were projecting from its surface. The difference in appearance could be due to some biochemical agent in the ooplasm which is released into the perivitelline space, influencing the corona cell morphology. One such agent might be a prostaglandin - another, hCG (44). It should be mentioned also that corona cells trapped in the perivitelline space can be mistaken under the light microscope for extruded polar bodies (107) or cytoplasmic fragments.

C-virus-like particles either budding from the microvilli or lying free in the perivitelline space were observed in some oocytes, but not in the early cleavage stages. Such particles, which had a diameter of slightly less than 100 nm, were bounded by a triple-layered membrane and enclosed a dark core with a less dark central mass in budding particles, or a homogeneous dark core in free-lying particles. The expression of virus-like particles is dealt with in greater detail in a later section.

Cortical granules, which induce in the zona a reaction which prevents polyspermia after fertilization (23,74), were located close to the cell membrane and also deep within the cytoplasm in GV oocytes. In preovulatory oocytes, the density of cortical granules near the cell membrane varied in different sections of an oocyte, but in some there was a rim of cortical granules in a single layer by the cell membrane and hardly any deep in the ooplasm. However, this was observed both in mature and in some immature oocytes lacking a GV. Thus, the location of cortical granules does not seem to be a reliable indicator of the maturation stage of an oocyte.

Cytoplasmic organelles were distributed throughout the ooplasm. Golgi complexes, which were not so prominent, appeared scattered in the cytoplasm. Each Golgi complex consisted of a few membranes surrounded by small vesicles. Rounded mitochondria with a few peripherally located crista were a dominant feature both in oocytes and in early cleavage stage ova. Elongated mitochondria with transverse crista were more frequently observed in later than in early cleavage stages. This change in the appearance of mitochondria is regarded as a sign of mitochondrial maturation (85). The smooth endoplasmic reticulum (SER) was closely associated with mitochondria and more so in maturing oocytes than in oocytes possessing a GV. In areas where profiles of SER were sparse, mitochondria were rare, while where profiles of SER were common, mitochondria tended to aggregate. In immature oocytes, profiles of SER were mostly small and irregular, while they were more often seen as distended sacs in preovulatory oocytes and in early cleavage stages. The SER, which is thought to participate in carbohydrate metabolism and steroid synthesis (40), seemed to be more active, due to its greater volume, in mature than in resting oocytes and the processes also seem to be energy demanding. Since the rough endoplasmic reticulum was sparse, protein synthesis was probably weak in oocytes and cleaving ova.

Membrane-bound vacuoles were observed in the centre of the ooplasm in immature oocytes but were uncommon in preovulatory ones. Some vacuolization is probably not a sign of degeneration (16,66,77), but numerous vacuoles in a few immature oocytes probably indicates imminent atresia. If this is true, 4 out of 5 immature oocytes would still have the potential to mature in vitro.

Multivesicular bodies were observed in oocytes possessing a GV, but hardly any in later maturation stages. These bodies were therefore probably involved only at a specific stage of maturation, and since they are part of the lysosome system (40), maybe at germinal vesicle breakdown. In cleavage stages, a type of granulated vesicle appeared. These structures resembled multivesicular bodies, though smaller. It may be that the same type of structure reappears to take part in nuclear breakdown at mitosis in blastomeres.

Mitochondria Vesicle (MV) complexes appeared in maturing oocytes and were numerous in cultured oocytes. The MV complex consisted of a single membrane-bound vesicle intimately surrounded by 5 to 20 rounded mitochondria. In oocytes possessing several MV complexes, aggregates of flattened tubuli of SER were also prominent. Usually, these aggregates were surrounded by a rim of one to three layers of mitochondria. Occasionally, a few small aggregates without surrounding mitochondria were observed in immature oocytes. It seemed that tubuli aggregation appeared first and that aggregates surrounded by mitochondria appeared at a later stage of maturation. The aggregates were probably related to MV complexes. In oocytes with many MV complexes and tubuli aggregates, nearly all the mitochondria clustered round these structures. The appearance of complexes in preovulatory oocytes could be explained by a process of maturation; the complexes may be required at a forthcoming fertilization and/or early cleavage. The only obvious difference between cultured and immediately fixed preovulatory oocytes was that aggregates of tubuli of SER and MV complexes were less numerous in the latter. This observation strengthens our opinion that the appearance of MV complexes is a sign of maturation.

Numerous MV complexes in some cultured oocytes might be a result of a continuous process of maturation, but as yet we know neither the optimal number of complexes, nor their function. The MV complexes were usually uniform in size in an oocyte, though occasional large complexes were observed. These might mistakenly be interpreted under the light microscope as pronuclei. The MV complexes were fairly common also in early cleavage stages, while only remnants of them were seen in later stages.

The nucleus was centrally located in blastomeres and centrally or peripherally in oocytes in the germinal vesicle (GV) stage. In some nuclei a dark nucleolus surrounded by chromatin was observed. The nuclear membrane displays regular dense spots which represent nuclear pores. In the GV stage, which is a resting stage, no material was released through these pores, nor was there any close association between the nuclear membrane and organelles in the ooplasm. In blastomeres, the outer leaflet of the nuclear membrane often formed outpouchings, some of which contained a dark body. These outpouchings appeared to be released into the cytoplasm; their function is still unknown.

In short, oocyte maturation was characterized ultrastructurally by a reduced number of multivesicular bodies and vacuoles, by an increase in the size of the endoplasmic reticulum, by an increased clustering of mitochondria around SER and by the appearance of mitochondria-SER aggregates and mitochondria-vesicle complexes (Fig. 4). Thus, in comparison with earlier investigations on maturing oocytes, new features characteristic of each stage of maturation were found in this study.

In cleavage stage ova, few microvilli were found on the cell membrane of the blastomeres. Elongated mitochondria were more frequent than in oocytes. MV complexes observed in preovulatory oocytes rapidly diminished in number during cleavage and only remnants were found in later cleavage stages.

Pathological features in oocytes and early cleavage stage ova:

Some oocytes, both immediately fixed and cultured, showed signs of degeneration. Degenerating oocytes were of two kinds, viz. atretic and pre-atretic oocytes. Atretic oocytes showed extensive vacuolization throughout the ooplasm, few or no microvilli on the cell membrane, and a condensed ground substance. Those oocytes which were obtained from small follicles and which were atretic had probably degenerated already early in the cycle, while pre-atretic immature oocytes, showing numerous vacuoles but otherwise a well preserved ooplasm, might have commenced degeneration shortly before collection.

Oocytes cultured for 2 to 3 days were either normal or atretic. It might be that most of the atretic oocytes had commenced degeneration already prior to collection. This is supported by our observation that two preovulatory oocytes (clinically classified), which were immediately fixed, were degenerating. Light microscopy is thus not always adequate for judging the condition of ova surrounded by cumulus cells.

Cytoplasmic fragments were found only in cleaved ova. The fragments, which were rather small, appeared early and persisted throughout cleavage. Since cleaving ova containing fragments show signs neither of degeneration (3) nor of slow development, these fragments were not regarded as a pathological sign. Furthermore, cleavage stage ova with fragments observed in

the light microscope have been replaced and have developed normally (46, 100,101).

It has been described by other investigators and by us that two or more nucleus-like structures can appear in a single blastomere, while another blastomere of the same egg possessed only one nucleus (46,62,77). It has been pointed out that, following prolonged in vitro culture, cleaving ova show an increasing frequency of nuclear abnormalities. These might be caused by the artificial culture environment (77). However, it might be that binucleate blastomeres are lethal and that remaining blastomeres replace them and accomplish a normal development (98,100).

Future prospects:

One of the clinical problems with IVF is that preovulatory oocytes quite often do not become fertilized despite a normal semen (89). Since the fertilization rate is higher in preovulatory oocytes incubated for 5 hours before addition of spermatozoa than in preovulatory oocytes incubated with spermatozoa immediately after recovery (91), one can assume that this difference is also reflected in some alteration of the oocyte ultra-structure. Maybe the number of MV complexes and aggregates of mitochondria-SER changes during this time and that these are needed in certain proportions for successful fertilization. This hypothesis is to be tested, as it might then be possible to determine the optimal time for fertilization.

In other, non-human mammals, a high rate of parthenogenesis has been reported at IVF of ova (13). It would be of interest to study controlled parthenogenetically developed "cleaving ova", since such cleaving ova could be a cause for implantation failure in an IVF program. It is important to be able to distinguish a developing egg derived from normal fertilization, from a parthenogenetically developed one.

The number of implantation failures after ER is high (90,100). This might be due to a failure in the egg or to a mechanical failure at replacement (100). The embryonic signals might be weak in IVF developed eggs and this could explain why a pregnancy more often occurs after replacement of several eggs since the total amount of the signal substance is then

greater and more powerful. If the signal substance is hCG or prostaglandin (51) - or some other as-yet undetected substance - it might be worthwhile to embed the egg in a clot (fibrin for instance) soaked with the signal substance, thereby increasing its potency and its prospects of implantation. The use of a fibrin clot at transfer was initiated in order to fix the egg at the site where it is deposited in the uterine cavity, thereby avoiding an ectopic pregnancy. The method has been tested in mice and parturition was achieved after replacement of eggs embedded in a fibrin clot (59).

EXPRESSION OF C-TYPE RETROVIRUS-LIKE PARTICLES IN OOCYTES AND FOLLICULAR FLUIDS

Retrovirus contains an enzyme, RT, which transcribes viral RNA to DNA. The reverse transcription - hence the name retrovirus - is essential for the propagation of a retrovirus (43,97). On a functional basis, retrovirus can be divided into two types - exogenous and endogenous. The exogenous viruses spread horizontally and can infect other cells. Endogenous viruses are transmitted vertically, i.e. they are inherited according to Mendelian laws (1) and have been detected as particulate form in organs involved in reproduction, fertilization and embryo genesis (106).

Morphologically, retroviruses can be classified into types A, B, C, and D. Type A particles are believed to be precursors of B and D particles. Types A, B and D particles differ morphologically from type C particles. The latter are easily observed as they are released from the cell membrane by a process of budding (94,97). The particles show a triple-layered cell membrane with an electron-lucent centre in budding particles and an electron-dense core in free-lying particles. The core contains the viral RNA, RT, the main core protein p30 and some other core proteins.

Particles similar to C-type virus have been detected in a few human tissues and in most animal tissues, including pre-implantation embryos. Virus expression in early cleavage stages in mice was found to be stage-specific and did not interfere with normal development. Thus, it was concluded that expression of C-virus-like particles is a normal event in the development of the egg (106).

At TEM analysis of oocytes, we observed particles with striking resemblance to C-type virus particles on the cell membrane. This encouraged us to confirm the observation by using detection methods for C-virus expression in oocytes and in follicular fluids. Several such methods are available (96,97) (Fig. 5):

- 1) detection of the particle by TEM;
- 2) detection of enzymatic activity, i.e. the presence of RT in follicular fluid;
- 3) detection of virus-related proteins, especially p30.

4) detection of viral RNA or its DNA copy.

In the present study, methods 1 and 2 were used.

RESULTS OF PRESENT INVESTIGATION AND DISCUSSION - Paper III

C-type like particles were observed by TEM in 15% of the oocytes, irrespective of their stage of maturation.

Reverse transcriptase activity was detected in 3 of 9 follicular fluids in this study, though in another study, including several hundred follicular fluids, we detected RT and virus-related protein (p30) in about 65% of the follicular fluids. Furthermore, retrovirus-related RNA has been detected in oocytes by in situ hybridization using a radiolabelled retroviral single-stranded DNA sequence as probe (56). Thus, we feel fairly confident that the particles originally observed on the cell membrane of the oocytes were C-virus particles.

It has been established that human DNA contains several sequences related to retroviruses (15) and the task now is to understand the function and implication of these sequences. For instance, endogenous viruses could affect differentiation either by functioning as differentiating genes or by affecting the transcription of other differentiating genes. It has also been suggested that endogenous viruses play a role in cell-to-cell communication at the acceptance of an egg by the host (37), though there is still no proof of a functional role in any species.

SPERM ATTACHMENT

Spermatozoa are required to undergo capacitation and acrosome reaction before they can fertilize an oocyte (23,47,). These events are needed for spermatozoa to pass through the layers surrounding the egg cell, viz. the cumulus cells, the corona radiata cells and the zona pellucida. Capacitation normally occurs in the oviduct within a few hours after coitus, though it can also be induced by incubating spermatozoa in culture media. Capacitation is a biochemical process which in turn permits the acrosome reaction to occur. The acrosome, which forms a cap around the anterior part of the sperm head, releases enzymes which allow the sperm to penetrate the layers surrounding the oocyte. The enzyme required to enable the spermatozoa to penetrate the surrounding layers of cumulus-corona cells is hyaluronidase and, for zona pellucida penetration, acrosin.

The zona pellucida is a non-cellular layer enclosing the oocyte. It is composed mainly of polysaccharides and glycoproteins. The zona pellucida has several roles, e.g. sperm recognition, attachment of spermatozoa, blocking of polyspermia, and protection of the egg (79). Species-specific sperm receptors are located on the zona surface (10) and, if the zona is removed, interspecies fertilization can be accomplished (105). The sperm-receptor sites can be inhibited by protease and by antisera and they are altered after fertilization, i.e. after the zona reaction, mediated by the cortical granula release, has occurred (41). In the mouse, three different glycoproteins have been found in the zona, one of which possesses the receptor activity (14). The exact counterpart in human oocytes is not known. However, a similar composition and role for receptor sites on the human zona pellucida is probable.

Human semen normally contains many morphologically abnormal spermatozoa (5,19,24,31,32,). In vivo, these are filtered off on their way to the site of fertilization, allowing almost only normal spermatozoa into the Fallopian tube (25). This mechanism is lacking in vitro and both normal and abnormal spermatozoa can interact with the oocyte. The interaction between human gametes has been investigated by light microscopy (65) and by TEM (52,76,80), though these techniques have certain limitations such as the light microscope's low resolution and the fact that only parts of a few spermatozoa can be analysed in any one section by TEM. Scanning electron

microscopy makes it possible to view the surface of the zona pellucida and to judge attached spermatozoa morphologically.

RESULTS OF PRESENT INVESTIGATION AND DISCUSSION - Paper IV

SEM examinations of the interaction between oocytes and spermatozoa revealed that usually, 10 to 50 spermatozoa attached to a preovulatory oocyte. However, occasionally more than a hundred spermatozoa were observed on a few non-ovulatory oocytes. It is therefore conceivable that there might be differences in sperm binding sites in and between immature and mature oocytes.

The number of spermatozoa needed to ensure fertilization in vitro has not yet been established, but a surprisingly small number - in spite of all the spermatozoa in the fertilization droplet - were in most cases found on each oocyte after incubation. It would seem that an oocyte has only a limited number of sperm attachment sites when mature and that attachment of several hundred spermatozoa is possible only on immature oocytes.

Morphologically abnormal spermatozoa only rarely attached to the zona pellucida, even though 30 to 40% abnormal spermatozoa, predominantly displaying amorphous heads, were present in the fertilization droplets. Some oocytes were almost denuded at collection, while others were surrounded by a cumulus mass or by several layers of corona cells. Although there was an easier access for spermatozoa to some oocytes, abnormal spermatozoa were not found in a greater proportion on these oocytes than on others.

Another way of judging the proportion of abnormal spermatozoa near the oocyte, is to view the surrounding corona cells - or rather the concavity formed by corona cells formerly facing the oocyte. In this cavity, numerous spermatozoa were observed. The proportion of abnormal spermatozoa among the corona cells was about the same as that found on the zona pellucida of the oocytes. There seems to be a selection of abnormal spermatozoa in between cumulus corona cells and also on the zona pellucida.

Future prospects:

Semen of low quality is a common cause of childlessness in couples. We

have sometimes observed a poor dispersion of the cumulus cells and that no spermatozoa attach to a preovulatory oocyte, especially when there is a large proportion of abnormal and a small proportion of motile spermatozoa in the semen. The total amount of hyaluronidase released from spermatozoa might be low in sperm samples with a large proportion of pathological spermatozoa and this might be the reason for the poor dispersion of the cumulus mass. As a consequence, the few normal spermatozoa in the sample might be unable to penetrate through the many layers of cells to reach the oocyte. In vitro, the cumulus mass surrounding the oocyte can be reduced in volume by adding hyaluronidase, (8), thereby shortening the distance that the shoal of morphologically normal spermatozoa and spermatozoa having a reduced motility must travel to reach the oocyte. Maybe this procedure could enhance the fertilization rate when the semen sample is of low quality.

PREIMPLANTATION ENDOMETRIUM

Regulation of the menstrual cycle:

Early follicular growth is a continuum and appears to be independent of gonadotropin stimulation (82). Most growing follicles regress, but due to a rise in FSH during the last days of the previous luteal phase, a group of follicles continue to grow, though normally only one of these will ovulate. During the early period of FSH-stimulated follicular growth, hardly any change is found in the plasma levels of gonadal steroids (Fig. 6). A rise in peripheral blood levels of oestradiol begins on approximately cycle day 6-7. FSH and oestradiol initiate granulosa cell proliferation. The number of gonadotropin receptors increases during follicular maturation. Oestradiol and FSH are responsible for the increase in the number of FSH receptors and FSH is responsible for induction of luteinizing hormone (LH) receptors.

During late follicular phase, the oestradiol level peaks and triggers the LH surge. The LH in turn acts on the LH receptors on granulosa cells and suppresses their inhibitory effect on oocyte maturation (50). Ovulation occurs about 28-32 hours after initiation of the LH surge.

Simultaneous with the decrease in the FSH level a few days before ovulation, due to the increasing levels of oestradiol, a wave of atresia of smaller and less differentiated follicles occurs. A slight increase in progesterone is seen during the LH surge before follicle rupture, followed by a rapid increase after ovulation. These effects are due to luteinization (LH effect) of the granulosa cells in the follicle.

The corpus luteum declines in activity 9 to 11 days after ovulation if conception and subsequent implantation of an hCG producing egg does not occur. The progesterone level reaches a maximum at the time for implantation and continues to rise if implantation occurs. Otherwise, the progesterone level drops sharply and menstruation starts a few days later. The life span of the corpus luteum, i.e. from ovulation to the onset of menstruation, is a fairly constant 14 days.

In an IVF program, stimulated cycles are almost exclusively used. The drugs used for ovarian stimulation are usually clomiphene citrate in combination with hCG or clomiphene citrate, human menopausal gonadotropin (hMG) and hCG.

Clomiphene citrate acts on the hypothalamus, resulting in increased production of gonadotropins. The rise in FSH stimulates a set of follicles to start growth and maturation. hCG has biological effects very similar to those of LH and induces ovulation about 36 hours after being administered at mid-cycle. hMG contains both FSH and LH, but the FSH-effect is the dominant one. hMG acts directly on the ovaries, resulting in growth of several follicles (82).

In our IVF programme, we use mainly clomiphene-hCG stimulated cycles. Clomiphene is given in doses from 50 to 150 mg usually from day 5 to day 9 in the menstrual cycle. Several follicles are usually induced to grow and plasma oestradiol is therefore high in late follicular phase. If hCG is not administered at mid-cycle, an endogenous LH surge occurs. To predict the time of ovulation, it is necessary repeatedly and at close intervals to analyse urine or plasma for LH. Since ovulation occurs about 28-32 hours after the onset of the LH surge and as this surge can occur at any time, an operating team must be on hand around the clock to aspirate the follicles shortly before natural ovulation.

Although oestradiol- 17β reaches a very high preovulatory plasma level in clomiphene-stimulated cycles, it is usually possible to regulate the time of ovulation before the endogenous LH surge has begun by injecting hCG. After the hCG injection, ovum pick-up must be performed within 34-36 hours. Several preovulatory oocytes - but even intermediate stages - are usually obtained at follicle puncture in clomiphene stimulated cycles (102). In the luteal phase, higher levels of plasma oestradiol and progesterone are seen than in unstimulated cycles, as several corpora lutea are then developing.

Clomiphene exerts an antioestrogenic effect during the period of administration. However, its effect may persist and a negative influence has in fact been seen on the cervical mucus and its permeability to spermato-

zoa at the time of ovulation. In the luteal phase, a persisting effect of the clomiphene treatment may affect the development of the endometrium. Thus, it is important to know whether the stimulation affects the endometrial surface epithelium differently in the luteal phase in comparison with that in unstimulated cycles.

Endometrial morphology:

The structural and functional changes of the endometrium are dependent upon the endocrine activity of the ovaries, i.e. predominantly oestrogen in the follicular phase and progesterone and oestrogen in the luteal phase. The first half of the endometrial cycle is concerned with growth and the second half with epithelial and stromal differentiation (64).

The endometrium consists of the surface epithelium, the uterine glands and the endometrial stroma. During the follicular phase, there is a two to three fold increase in thickness of the endometrium. Mitoses are numerous and glands increase in number and length. During the luteal phase, there is a further thickening of the endometrium. This is largely attributed to oedema of the stroma and to accumulating secretion in the uterine glands. Two days after ovulation, subnuclear vacuolization of the gland epithelium becomes prominent. The vacuoles decrease in size after another 2 days. The location of the nucleus of the cells also changes during this period. The alterations in the glandular epithelium have been used to date the first half of the luteal phase, while stromal characteristics have been used to date the second half (64). In late luteal phase, a pseudodecidualization occurs. This is a change in which stromal cells are transformed into large decidual cells rich in glycogen. If implantation of an egg occurs, a true decidua is formed.

The surface epithelium is a single cell layer composed of ciliated cells and non-ciliated cells (secretory cells). The cilia beat towards the lumen in glands and towards the cervix-vagina on the surface epithelium. Secretory cells are active in both follicular and luteal phases.

Transmission electron microscopy has confirmed the light microscopic findings (103). Furthermore, it has revealed that the number and length of microvilli increase towards ovulation and that apical protrusions, both

with and without glycogen granules, appear as the time for implantation approaches.

Several scanning electron microscopic studies have demonstrated changes in the follicular phase in comparison with the luteal phase (7,26,27, 28,33,34,42,48,60,99). The hormonal influence is best demonstrated in a model system in postmenopausal women (53,54). Thus, it was found that oestrogen induces formation of both ciliated and non-ciliated cells and also small apical protrusions. Gestagen treatment did not affect the endometrial surface structure, whereas sequential treatment with oestrogen and gestagen resulted in a surface structure similar to that which characterizes a premenstrual uterine surface, i.e. an epithelium possessing ciliated and non-ciliated cells and large morula-like apical protrusions on the non-ciliated cells.

In women with normal menstrual cycles, the surface epithelium is re-established within 5 days after menstruation and non-ciliated and ciliated cells have developed. Apical protrusions on the non-ciliated cells appear in the luteal phase. However, inconsistent results have been found at SEM investigations when examining changes in the luteal phase, possibly because the dating of the biopsies was inexact. Therefore, it had not been established whether there is a day-by-day change in the surface epithelium, from ovulation to the time for implantation. This question is crucial if implantation only is compatible with a specific surface structure and if the use of ovarian stimulation affects this structure.

Following IVF, the cleaving egg is usually replaced into the uterine cavity of the oocyte donor 2 days after ovum pick-up and fertilization. In vivo, the egg does not reach the womb until approximately day 3 to 4 after ovulation (18). The asynchrony in vivo versus in vitro may have an effect on implantation.

RESULTS OF PRESENT INVESTIGATION AND DISCUSSION - Paper V

For indicators of changes on the postovulatory surface epithelium, the frequency and appearance of ciliated cells, non-ciliated cells and apical protrusions in the groups of biopsies were examined and compared.

Ciliated and non-ciliated cells dominated the surface epithelium. The ciliated cells appeared singly and possessed cilia which were of almost equal length and number on each of the cells. The non-ciliated cells had microvilli of varying length and number. The lengths of microvilli differed even on cells in the same field of vision. The ratio of ciliated to non-ciliated cells varied greatly in different areas, but this variation was seen on any day within the first week after ovulation and irrespective of whether or not ovarian stimulation had been induced. Thus, no differences in either ciliated or non-ciliated cells were found between unstimulated and stimulated women within the first week after ovulation.

Apical protrusions were found in all specimens, irrespective of the day, or of whether stimulation was induced. Sometimes large areas with protrusions were observed. These protrusions, in groups or in large areas, may be important for ovum attachment. Since it has been shown that in mice these protrusions formed the closest contact with the trophoblast cells at implantation (12), they may be adhesive (55).

Although plasma hormone levels of oestradiol and progesterone differed greatly from day to day and during any specific day, in unstimulated and stimulated cycles post ovulation, this seemed not to affect the structures of the surface epithelium. Further, the antioestrogenic effect of clomiphene (total 250 mg) was not reflected in any differences in the structures of the surface epithelium in comparison with those of unstimulated cycles.

To conclude, scanning electron microscopy of the uterine epithelium, carried out in unstimulated and stimulated cycles from day 1 to day 7 after ovulation, revealed no alterations in the morphology of the surface epithelium, from ovulation to the time for implantation, nor were there any marked differences whether stimulation had been induced or not. Consequently, the surface may be ready to accept an egg at any time within a range of days post ovulation and the crucial time point for implantation may be governed by other changes in the endometrium or by the developmental stage of the egg.

Future prospects:

It is not known what causes the blastocyst to attach to the uterine epithelium. Differences in surface properties of the epithelial cells and of the trophectoderm could be one factor. Surface properties can be evaluated in various ways. Among the morphological methods, the use of colloidal iron (for surface charge) and lectin-binding sites (for carbohydrate residues) can be mentioned (58). By using these techniques, it might be possible to get some indication whether the endometrial surface properties change during the peri-implantation period and whether ovarian stimulation interferes with this change.

Figure 1. MEIOSIS, FERTILIZATION AND CLEAVAGE

(A) Prophase of meiosis to the first meiotic arrest.

Oogonia are produced mitotically during early fetal life. Meiosis begins at the 5th month of fetal life and progresses to the prophase of the first meiotic division. At birth, the oocytes have entered a resting stage (GV stage) which may persist for several decades before the oocyte is reactivated and meiosis is resumed.

Stages of prophase of meiosis: 1) Leptotene. Single-strand chromosomes (23 chromosome pairs). 2) Zygotene. Pairing of chromosomes (diploid). 3) Pachytene. Splitting of chromosomes (tetraploid), crossing over. 4) Diplotene. Resting oocyte.

(B) Resumption of meiosis to the second meiotic arrest.

The time required from GV breakdown to the second meiotic arrest, at which stage the oocyte is ovulated, is about 36 hours.

First meiotic division: 1) Breakdown of GV membrane. 2) Formation of first meiotic metaphase spindle. 3. Extrusion of the first polar body. 4) Formation of second meiotic spindle and arrest of meiosis (diploid).

(C) Fertilization and pronuclei formation.

The time required for the fertilizing spermatozoon to penetrate the zona pellucida is about 20 min. Cortical granules preventing polyspermy are triggered for release by the fertilizing spermatozoon. Meiosis is resumed and the second polar body is extruded within one hour of fertilization. The female and male pronuclei can be observed 12-24 hours later.

Fertilization, second meiotic division, pronuclei formation: 1) Fertilization and resumption of second meiotic division. 2) Second meiotic division completed and second polar body extruded (haploid). Decondensation of the sperm head begins (haploid). 3) Formation of female and male pronuclei. 4) The pronuclei move towards the centre. The chromosomes (23+23) restore their diploid number.

(D) First mitotic cleavage to blastocyst formation.

1) Beginning of first mitotic cleavage (46 chromosomes). 2) Two equal-sized, nucleated blastomeres are formed approximately 25 to 40 hours after fertilization. 3) Mitotic cleavage proceeds from the 2-cell stage to the 4-cell stage. The number of cells is doubled at each cleavage while the blastomeres are still enclosed within the zona pellucida. The egg reaches the uterine cavity on the 3rd to 4th day following ovulation. 4) The zona pellucida has been shed and trophoblasts and an inner cell mass (embryonic cells) have developed. This stage is called the blastocyst stage. Implantation of the blastocyst takes place on the 6th to 7th day following ovulation.



n : total amount of DNA; cr : chromosome; nu : nucleus; zp : zona pellucida; pb : polar body; sp : sperm; pn : pronucleus; bl : blastomere; bic : blastocyst.

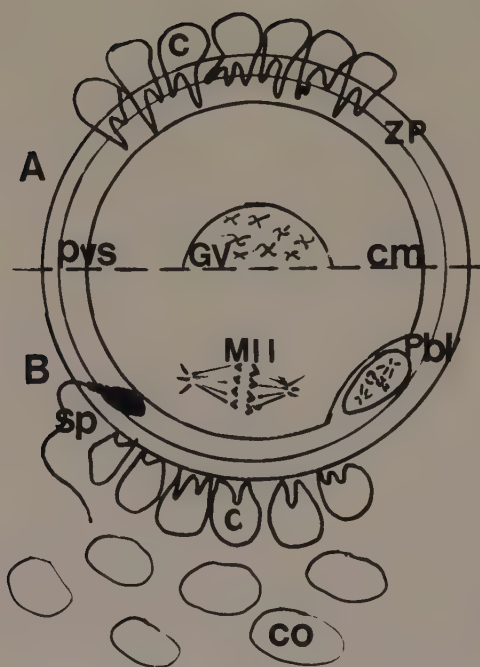


Figure 2. SCHEMATIC DRAWING OF THE OOCYTE

(A) The immature oocyte is surrounded by corona cells (c) and the zona pellucida (zp). The gap between the zona pellucida and the cell membrane (cm) is the perivitelline space (pvs). A resting oocyte contains a germinal vesicle (GV).

(B) The mature oocyte is surrounded by a dispersed mass of cumulus oophori cells (co) and loosely attached corona cells. The first polar body (pb) has been extruded and meiosis is arrested at the second meiotic metaphase (M II). A spermatozoon (sp) has penetrated the zona pellucida and is approaching the oocyte cell membrane. The fertilizing spermatozoon triggers the release of cortical granules and also the second meiotic division, i.e. extrusion of the second polar body.

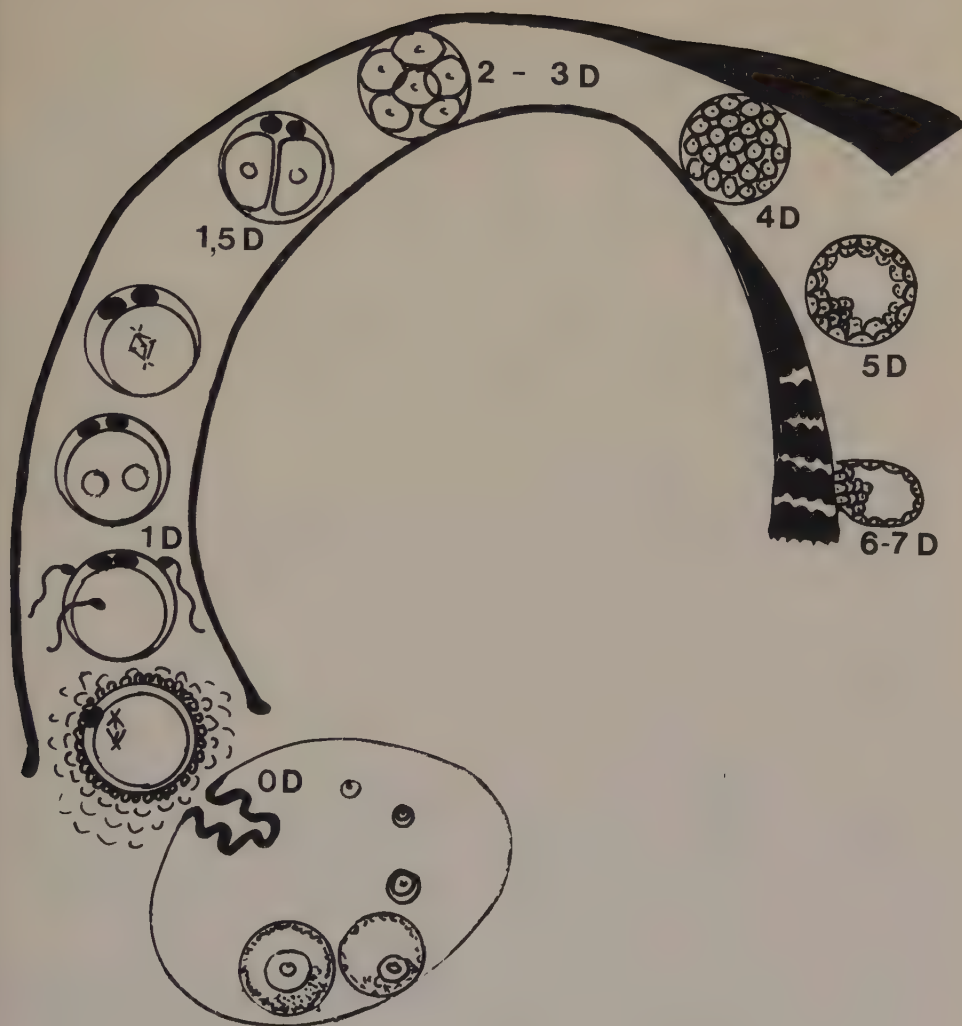


Figure 3. SCHEMATIC DRAWING OF OVULATION, FERTILIZATION, CLEAVAGE AND IMPLANTATION OF AN EGG IN VIVO
D: Days

Figure 4. ULTRASTRUCTURAL CHARACTERISTICS OF OOCYTES AND EARLY CLEAVAGE STAGES

(A) Resting oocyte in the germinal vesicle (GV) stage.

The nuclear membrane contains pores. A dark nucleolus was occasionally seen in the nucleus. Vacuoles (va) were fairly frequently observed. Rounded mitochondria (mi) with a few crista were scattered in the cytoplasm and usually associated with irregular profiles of the smooth endoplasmic reticulum (SER). The Golgi apparatus (g) was not a prominent feature in oocytes. Cortical granules (cg) were located by the cell membrane and deep in the cytoplasm. Microvilli were seen extending from the cell membrane into the perivitelline space (pvs). Virus-like particles were observed in some oocytes, regardless of their stage of maturation.

(B) Immature oocyte without a GV.

The association between SER and mitochondria was pronounced and in areas where no profiles of SER were seen, mitochondria were sparse. Multivesicular bodies were rarely observed. Aggregates of SER tubuli were occasionally seen. Lysosomes (ly) were observed in many oocytes, irrespective of their maturation stage.

(C) Cultured preovulatory oocyte.

Vacuoles were infrequent. SER often appeared as rounded, distended sacs. Aggregates of SER tubuli surrounded by mitochondria, and vesicles surrounded by mitochondria (MV) were fairly numerous. Cortical granules were usually located close to the cell membrane.

(D) Cleavage stages.

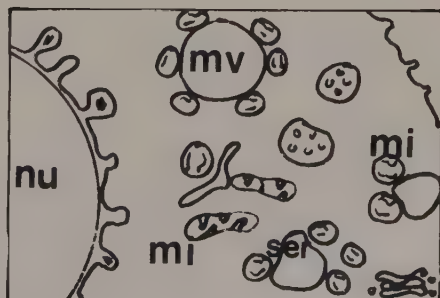
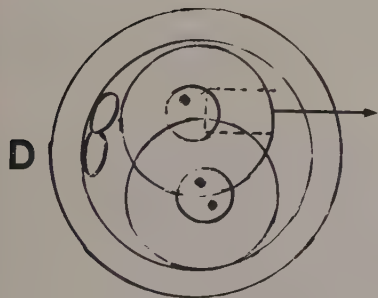
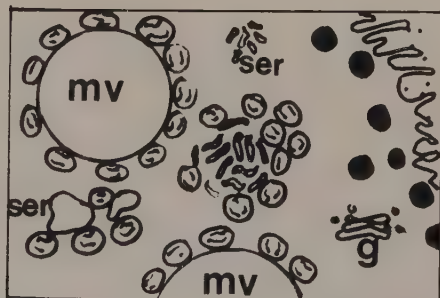
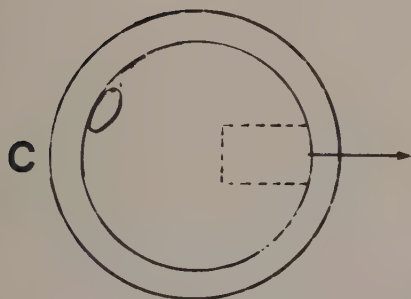
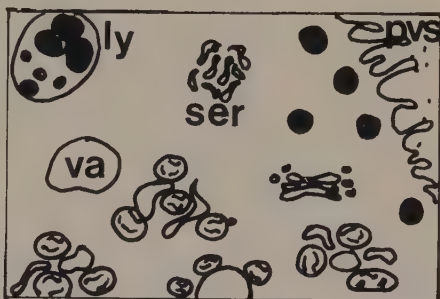
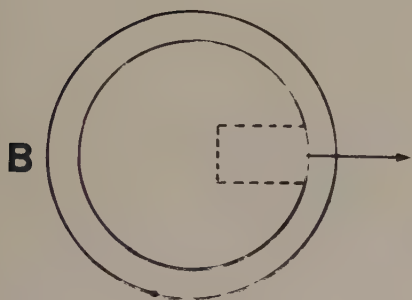
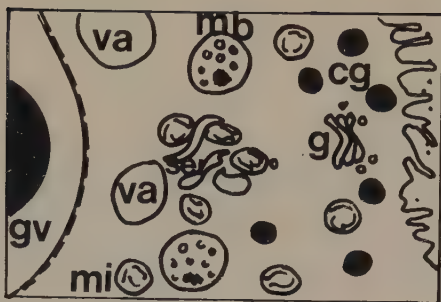
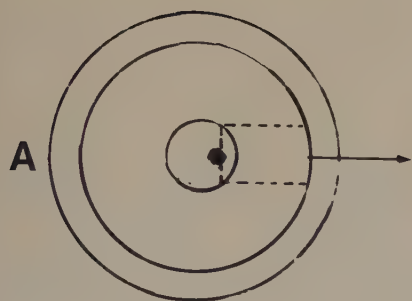
The outer leaflet of the nuclear membrane formed outpouchings into the cytoplasm. Elongated mitochondria with transverse crista were seen. MV complexes were seen in early cleavage stages, while only remnants were seen in later stages. Microvilli on the cell membrane were few and no virus-like particles were observed.

- The mitochondria provide the cell with adenosine triphosphate (ATP) to be used for energy demanding processes. The number of crista within the mitochondria reflects their state of activity. Mitochondria are also involved in steroid hormone production. In this process, some steps occur in the cisterna of the SER and others in the mitochondria. SER and mitochondria are then closely associated with each other.

- The Golgi apparatus consists of a system of flattened cisterna arranged parallel to each other. Its functions include storage, modification and pacing in vesicles of secretory products.

- The SER is a network of anastomosing tubuli. Its channels serve as routes for transport of materials between various regions of the cytoplasm or between cytoplasm and nucleus. SER is involved in lipid and steroid synthesis and in regulation of calcium ions.

- Lysosomes are membrane-enclosed bodies which function as storage vesicles for digestive enzymes. Multivesicular bodies belong to the lysosome system.



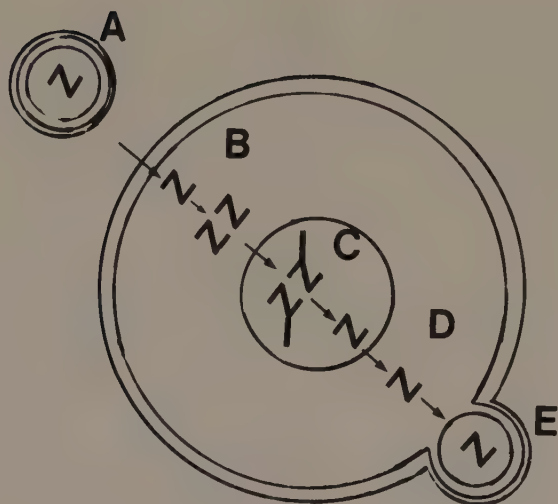


Figure 5. SCHEMATIC DRAWING OF THE LIFE CYCLE OF THE TYPE-C RETROVIRUS AND METHODS FOR ITS DETECTION

C-virus life cycle:

The virus particle contains viral RNA (A) which is transcribed by the enzyme reverse transcriptase (B) to DNA before it can be integrated into the cellular DNA (C). DNA is transcribed to viral RNA which is released into the cytoplasm (D). The virus particle forms buds at the cell membrane (E) and is then released from the cell.

Methods for detection of C-virus:

(A) Free-lying particle: - transmission electron microscopy - reverse transcriptase - virus protein (p30) - viral RNA.

(B and D) Free viral RNA and protein in the cytoplasm: - reverse transcriptase - virus protein - in situ hybridization of viral RNA - purification of viral RNA.

(C) Integrated proviral DNA: - hybridization.

(E) Budding particle: - transmission electron microscopy.

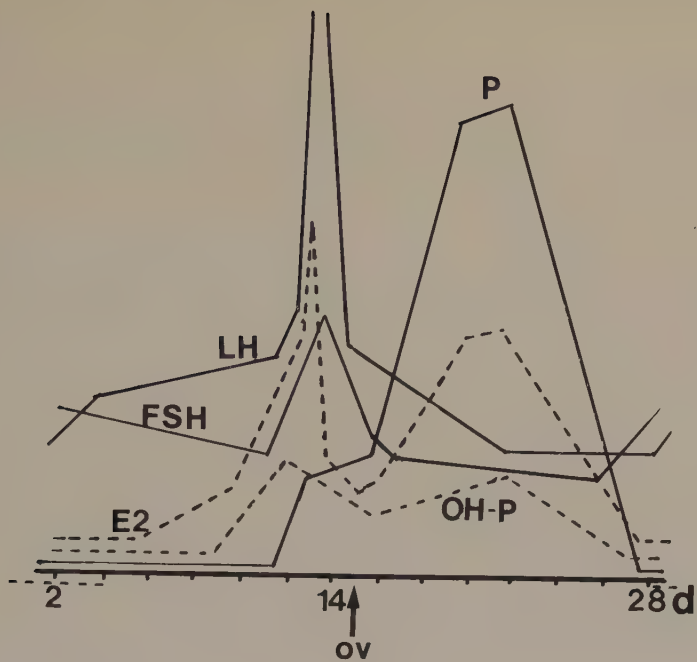


Figure 6. RELATION BETWEEN PITUITARY GONADOTROPINS AND OVARIAN STEROIDS IN THE NORMAL MENSTRUAL CYCLE (82)

LH: Luteinizing hormone
 FSH: Follicle-stimulating hormone
 E₂: Oestradiol-17 β
 P: Progesterone
 OH-P: 17-hydroxy progesterone
 ov: Ovulation

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